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Isolation and Characterization of Fungal Chitosan from Malaysian Isolates

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Chitosan from *Cunninghamella* sp. A1, *Absidia butleri* 2A1, *Rhizopus* sp UKM21 and *Mucor* sp UKM13 was isolated and characterized in this study. The effect of medium and nitrogen sources on the yield of chitosan was investigated. The amount of chitosan produced by these isolates was higher than previously reported, with *Cunninghamella* sp. A1, *Absidia butleri* 2A1, *Rhizopus* sp UKM21 and *Mucor* sp UKM13 producing 2.59 ± 0.5, 2.20 ± 0.25, 2.05 ± 0.30 and 1.96 ± 0.4 g/L chitosan respectively. The highest yield was obtained in Yeast-Peptone-Glucose (YPG) medium supplemented with peptone (range 0.82 - 2.92 g/L). In addition to affecting yield, these parameters influenced the degree of deacetylation (DD) and molecular weight (MW) of the fungal chitosan. The DD and MW for the fungal chitosan isolated in this study was between 80 - 95% and 5.1 - 35.9 x 10^4 Dalton respectively. Since the chitosan isolated had high DD and LMW, we tested its ability as an antimicrobial agent. The disc diffusion and Minimum Inhibitory Concentration (MIC) studies indicate that the fungal chitosan had higher inhibitory effects on Gram positive bacteria compared to the Gram negative organisms tested in this study. The fungal chitosan also showed bacteriocidal effects on *Candida* spp.

Key words: Chitosan, fungal, antimicrobial, bacteriocidal, degree deacetylation (DD), molecular weight (MW).

Chitosan [poly (ß-(1-4)-2amino-2-deoxy-D-glucose)] is a natural cationic and biodegradable polysaccharide derived from chitin[7,10]. The commercial variety is available through partial deacetylation of chitin (poly N-acetyl-D-glucosamine) from crustacean shells.

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Much research has been conducted on the isolation and application of crustacean chitosan. Amongst the widely studied applications of chitosan are as food preservatives, pharmaceutical products, flocculent in water treatment and resistance inducers in the agriculture industry[19,21]. Over the last decade, researchers have started looking at fungi as a source of chitosan. However chitosan is less commonly found in living organisms than chitin and can be found in the cell wall of certain groups of fungi, particularly Zygomycetes[2]. Therefore, Zygomycetes have become an alternate source in the extraction of chitin and chitosan[11]. Compared to the crustacean chitosan, the physicochemical

properties of fungal chitosan can be manipulated and standardised by controlling the parameters of fermentation[16,22] and other factors, including the strain of fungi used and the extraction protocol[1,5].

There are various applications of chitosan that range from the high value low volume products to the low value high volume output. The most valuable use of chitosan is in the biomedical and pharmaceutical applications. These applications would include antimicrobial activity, hypolipidemic and hypocholesterolimic applications, wound dressings, gene replacement therapy, drug delivery vehicle, osteoinduction, artificial kidney membrane, surgical sutures and artificial skin[7,21]. As the biomedical and pharmaceutical products are the most valuable, thence we decided to focus on the production, characterization and biomedical application of chitosan extracted from our local fungal isolates. In this paper we report the characterization of all the four fungal chitosan and the antimicrobial properties of *Cunninghamella* sp A1 and *Absidia butleri* 2A1 derived chitosan.

MATERIAL AND METHODS

Chitosan production

Spores from 5-7 day PDA plates of *Cunninghamella* sp. A1, *Absidia butleri* 2A1, *Rhizopus* sp UKM21 and *Mucor* sp UKM13 were used to generate spore suspensions. The spores (inoculum size $1x10^7$) were inoculated aseptically into autoclaved (110°C for 10 minutes) growth medium. The fungi was grown in two types of growth medium, a complex medium, YPG (Yeast Peptone Glucose) (10 g/L of glucose, 3 g/L of yeast extract, 10 g/L of peptone and 1 mL trace element at initial pH 5.5) and a defined medium, TVB (glucose 20g/L; $(NH)_4SO_4$ 1.4g/L; KH_2PO_4 2 g/L; $CaCl_2$ 0.3g/L; $MgSO_4.7H_2O$ 0.3g/L; molybdic acid 85% 0.01g/L and 1mL/L trace elements (per 500mL: $FeSO_4.7H_2O$ [5g], $ZnCl_2$ [1.66g], $CoCl_2.6H_2O$ [2g], $MnSO_4.7H_2O$ [1.96g] and hydrochloric acid 12 M [10mL]). These mediums were supplemented with either organic (urea, peptone and soybean) or inorganic (ammonium sulphate) nitrogen sources. Fermentation was carried out in 5L fermenter with 1L medium maintained at 30°C with aeration for 72 hours. The mycelia was then harvested, and chitosan was extracted from the mycelium using the White *et al.* (1979) methodology[24].

Chitosan Characterization

Degree of deacetylation: The degree of deacetylation was determined using first derivative UV-spectrophotometry (Cary 100 UV-Vis). Solution of 10mg/L -100mg/L of GlcNAc (N-acetylglucosamine) in 0.01M acetic acid was prepared to obtain the calibration curve. Forty milligrams (mg) of chitosan was diluted in 0.01M acetic acid and read spectrophotometrically at 197 nm. Degree of deacetylation of the chitosan samples was determined by the calculation of the glucosamine percentage[23].

Molecular weight

Molecular weight was determined through viscometric analysis. Chitosan (0.25%) samples were prepared in 1% acetic acid (20mL). The relative viscosity, ?, of chitosan samples was measured using a Ubbelohde capillary viscometer 1B at 25°C. Specific viscosity was determined using:

$$\eta_{sp} = (\eta_{solution} - \eta_{solvent})/ \eta_{solvent}$$

Intrinsic viscosity, [η] was defined as reduced viscosity, ηred, value was extrapolated to a chitosan concentration (C [g/mL]) of zero where $[\eta] = (\eta_{sp}/C)c\text{-}0 = (-\eta_{red})\ c\text{-}0$. Average molecular weight was calculated based on the Mark-Houwink equation: $[\eta] = KMav$, where K and a are dependent on the degree of deacetylation of chitosan (Table 1) [8].

Lightness

The chitosan sample was put in a transparent petri dish (35×10 mm). Color of chitosan was measured using a Minolta spectrophotometer (Model CM-508d) which was standardized with a calibration white plate (X= 86.95, Y= 91.82, Z= 98.93; L*= 96.75, a*= -0.18,

Table 1. The Value of K and a (depend on the degree of deacetylation)

Degree of Deacetylation	K value	a value
69	0.104×10^3	1.12
84	1.424×10^3	0.96
91	6.589×10^3	0.88
100	16.80×10^3	0.81

b*= -0.24). L*, a*, b* values indicate lightness, redness (negative a* value: greenness), and yellowness (negative b*: blueness), respectively.

Antimicrobial assays

The bacteria cultures were grown in Brain Heart Infusion (BHI) liquid medium at 37 °C. After 6 h of growth, each microorganism, at a concentration of 10^6cells/mL, was inoculated on the surface of Mueller-Hinton agar plates. Subsequently, filter paper discs (6 mm in diameter) saturated either with different concentrations of chitosan (50 µL) were placed on surface of each inoculated plate. To evaluate the efficiency of the methodology, each chitosan preparation at different concentrations was inserted simultaneously in a hole made (50 µL) in new plates. The plates were incubated at 37 °C for 24 h. After this period, it was possible to observe the inhibition zone. Overall, cultured bacteria with halos equal to or greater than 7 mm were considered susceptible to the chitosan preparations made in acetic acid. Zero point five percent (0.5%) acetic acid was used to dissolve the various concentrations of chitosan (2, 4, 6, 8 and 10 mg/mL). The control was 0.5% acetic acid and it showed no inhibitions in the preliminary studies.

The antimicrobial activity of those showing positive zoning effects were identified for the Minimal Inhibitory Concentration (MIC) assays. Bacterial samples were grown in nutrient broth for 6 h. One hundred µL of 10^6 cells/mL was inoculated in tubes with nutrient broth supplemented with different concentrations (2, 4, 6, 8 and 10 mg/mL) of chitosan. These cultures were incubated at 37 °C for 24 h and the MIC of each sample was determined by measuring the optical density at 620 nm spectrophotometerically. The sample readout was compared with the non inoculated nutrient broth.

Statistical analysis

Student t-test was used to analyse data between groups and one-way ANOVA among groups. Values of $p<0.05$ were considered as significant.

RESULTS

Source of chitosan

Fungal isolates were obtained from screening soil and food sources in Malaysia. All these isolates that were from the Zygomycete family were subjected to a preliminary screening of chitin-chitosan content of cell wall before a short-listed group of fungi were use in the study of fungal chitosan production. In this study *Absidia*, *Cunninghamella*, *Rhizopus* and *Mucor* were selected for further study in the production of chitosan. Table 2 provides the value of chitosan derived from 1L Yeast-Peptone-Glucose cultures of the abovementioned fungi.

Table 2. Chitosan Extracted Per Liter YPG Medium

Chitosan obtained g/L	*Cunninghamella* sp A1	*Absidia butleri* 2A1	*Rhizopus* sp UKM21	*Mucor* sp UKM13
	2.59 ± 0.5	2.120 ± 0.25	2.05 ± 0.30	1.96 ± 0.4

Characteristic Analysis of Chitosan

The fungal chitosan derived from this study were compared against the characteristics of the commercial crustacean chitosan. As the application of chitosan would be dependent on the degree of deacetylation and molecular weight of the chitosan, parameters that affected these values were studied. The quality of the chitosan obtained in this study was measured through the degree of deacetylation, molecular weight, and lightness values.

Effect of Nitrogen and Carbon on Chitosan Produced

From our previous research on *Absidia butleri* dr and *Rhizopus oligosporus* with glucose, fructose, sucrose, maltose, and lactose at 1-3% concentration as carbon source, glucose produced the best results at 2%, followed by fructose (2%), sucrose (2-3%) and maltose (1%) [12,13,14]. The best carbon source in all the studies we have conducted on chitosan production previously and in the current study is glucose. Therefore in this study

we used 2% glucose as the carbon source and sought to determine a suitable nitrogen source. Table 3 provides the amount of chitosan obtained with different nitrogen sources for all four fungal chitosans. In this study, peptone was found to be the best nitrogen source in chitosan production for these isolates followed by urea, ammonium sulfate and soybean.

Other then the carbon and nitrogen content, the type of medium used also influences the yield of chitosan. As seen in Table 3, YPG produced a significantly higher yield ($p<0.05$) of chitosan compared to TVB. YPG is a nitrogen rich medium where its source of nitrogen is from both the Yeast and Peptone content in the medium. YPG is able to produce good growth yield without any supplementation of additional nitrogen sources. However, even when supplemented with nitrogen sources, TVB could not produce growth and chitosan yield that was comparable to YPG (Table 3). The yield of chitosan was significantly lower ($p< 0.05$) in TVB as compared to YPG (Table 3).

Degree of Deacetylation (DD) and Molecular Weight (MW)

The degree of deacetylation obtained from our fungal chitosan was 80 - 95%, which is relatively higher than that of commercial chitosan. The highest degree of deacetylation (88-95%) was obtained with urea followed by peptone (87-90%), soybean (84-87%) and $(NH_4)_2SO_4$ (84-85%) (Table 4). The molecular weight of fungal chitosan observed in our studies was between 5.1 - 35.9 x 10^4 Da (Table 4). Previous studies have shown

Table 3. Chitosan Production Using Different Growth Medium (YPG and TVB) Supplemented with Different Nitrogen Sources at 30°C with Aeration. Results are as obtained with glucose 2%

Medium	Nitrogen Source	Yield of Chitosan (g/L)			
		Cunninghamella sp A1	*Absidia butleri* 2A1	*Rhizopus* sp UKM21	*Mucor* sp UKM13
YPG[a]	Peptone	2.92	2.5	1.97	0.82
	Urea	2.49	2.05	1.55	0.77
	$(NH_4)_2SO_4$	2.35	1.76	1.03	0.34
	Soy bean	1.9	0.67	0.49	0.11
TVB[b]	Peptone	0.89	0.61	0. 60	0.77
	$(NH_4)_2SO_4$	0.41	0.29	0.09	0.08
	Urea	0.69	0.56	0.66	0.54

[a] & [b] are significantly different

Table 4. Comparison of Fungal Chitosan and Commercial Chitosan Properties in Different Growth Medium (k & a are coeffiecent related to the Ubbhelohde tube & Degree of Deacetylation in Table 1)

Sample	Degree of Deacetylation(%)				Molecular weight (10^4 Da)			
	C	A	R	M	C	A	R	M
YPG peptone	90	87	88	88	29.3	22.7	26.5	20.5
YPG urea	95	92	88	89	35.9	26.3	29.7	21.2
YPG $(NH_4)_2SO_4$	85	84	84	84	22.4	17.8	16.5	17.9
YPG soy bean	84	87	85	86	13.4	17.3	15.1	16.5
TVB peptone	82	85	85	83	8.9	5.1	7.3	6.3
TVB urea	84	87	86	89	7.8	6.6	5.6	7.1
TVB $(NH_4)_2SO_4$	82	86	84	87	5.4	5.8	6.2	5.3
Commercial chitosan	84	234.6						

C- *Cunninghamella* sp A1; A - *Absidia butleri* 2A1; R - *Rhizopus* sp UKM21; M- *Mucor* sp UKM13.
MW in TVB and YPG are significantly different

the value of chitosan MW to be 3 x 10^4 Da - 1.4 x 10^6 Da (1, 17). This study however found that the average molecular weight for chitosan extracted from fungi grown on complex medium (YPG) was higher ($p<0.05$) (Table 4).

The type of fungi was also found to have an influence on the colour of the chitosan produced. Lighter coloured fungal mycelia produced lighter coloured chitosan (Table 5). The lighter coloured mycelia of *Rhizopus* sp. produced lighter coloured chitosan in comparison to the yellowish mycelia of *Absidia* sp. (Table 3). In addition, the colour intensity of the growth medium on which the fungi is cultured also affects the colour of the chitosan produced. YPG had higher colour intensity compared to TVB and therefore produced darker coloured mycelia, which in turn produced darker coloured chitosan (Table 5).

Antimicrobial Effect: Inhibitory and Bacteriocidal Effect of Chitosan

Table 5. Lightness Values, L for Chitosan Extracted from Fungi Grown on Different Growth Media

Fungus	YPG -L	TVB - L
Cunninghamella A1	[a]76.06[c]	[b]79.70[c]
Absidia sp. 2A1	[a]68.88[d]	[b]78.61[d]
Rhizopus sp. UKM21	[a]78.61[e]	[a]79.59[f]
Mucor sp UKM13	[b]82.05[e]	[b]83.77[f]

[a-b] Mean values in the same row bearing different letters are significantly different
[c-f] Mean values in the same column bearing different letters are significantly different

The inhibitory effect of these chitosan was examined against Gram positive and negative bacteria using 2.00 -10.00mg/mL chitosan concentration. Chitosan produced in peptone supplemented YPG medium for two fungal isolates; i.e *Cunninghamella* sp A1 and *A. butleri* 2A1 were used in the antimicrobial studies (Table 6A, 6B, 7A and 7B - The DD and MW of these chitosan are as in Table 4).

Table 6A. Antimicrobial Effects Against Gram Positive Bacteria (Chitosan from *Cunninghamella* sp)

Organism	% Inhibition at concentration (mg/mL)					% activity under thin pad	
	2	4	6	8	10	Inhibitory activity	Bacteriocidal activity
Staphylococus aureus (16)		11	87	92	94	100	86
Bacillus cereus(7)		71	92	100		100	92
Pseudomonas aerogenes(6)			20	100		100	0
Enterococcus aerogenes(5)			9	100		100	0
Candida spp (10)		6	33	44	89	100	100

Table 6B. Antimicrobial Effects Against Gram Positive Bacteria (Chitosan from *Absidia butleri* 2A1 sp)

Organism	% Inhibition at concentration (mg/mL)					% activity under thin pad	
	2	4	6	8	10	Inhibitory activity	Bacteriocidal activity
Staphylococus aureus (16)		33	78	95	88	100	80
Bacillus cereus(7)		69	87	100		100	74
Pseudomonas aerogenes(6)			32	100		100	0
Enterococcus aerogenes(5)			24	98		100	0
Candida spp (10)	21	39	54	91		100	86

Note: Figures in bracket are the number of isolates examined
% inhibition calculated on basis of the number of isolates exhibiting inhibition i.e. halo formation and lack of turbidity
Inhibition zones (halos) of 7mm or above is taken as susceptible to chitosan

Table 7A. Antimicrobial Effect Against Gram Negative Bacteria (Chitosan from *Cunninghamella* sp)

Organism	% Inhibition at concentration (mg/mL)					% activity under thin pad	
	2	4	6	8	10	Inhibitory activity	Bacteriocidal activity
Escherichia coli (21)		25	63	90	85	92	67
Klebsiella spp. (28)		41		96	78	88	50
Enterobacter spp. (5)		40	76	100	80	96	40
Serratia spp,(19)		20	39	53	45	58	45
Pseudomonas aeroginosa (25)			76			88	35
Acinetobacter spp. (5)				96	88	94	40

Table 7B. Antimicrobial Effect Against Gram Negative Bacteria (Chitosan from *Absidia butleri* 2A1 sp)

Organism	% Inhibition at concentration (mg/mL)					% activity under thin pad	
	2	4	6	8	10	Inhibitory activity	Bacteriocidal activity
Escherichia coli (21)		19	52	81	90	95	50
Klebsiella spp. (28)		39		82	96	96	48
Enterobacter spp. (5)		40	60	80	76	98	60
Serratia spp,(19)		15	31		53	58	54
Pseudomonas aeroginosa (25)			65			88	40
Acinetobacter spp. (5)				88	76	95	29

Note: Figures in bracket are the number of isolates examined
% inhibition calculated on basis of the number of isolates exhibiting inhibition i.e. halo formation and lack of turbidity
Inhibition zones (halos) of 7mm or above is taken as susceptible to chitosan

Table 6 and 7 are the results of the antimicrobial studies conducted on both Gram positive and Gram negative bacteria. Comparison between the data in Table 6 (A & B) and Table 7 (A & B) shows that the fungal chitosan is affective against Gram positive bacteria. The best inhibitory dosage as determined through this study is 8mg/mL where *Bacillus cereus*, *Pseudomonas aerogenes* and *Enterococcus aerogenes* show 100% inhibition (Table 6A and 6B). Chitosan also exhibited bacteriocidal affects on *Candida* spp (Table 6A).

DISCUSSION

The yield of chitosan obtained in this study from *Cunninghamella* sp. A1, *Absidia butleri* 2A1, *Rhizopus* sp UKM21 and *Mucor* sp UKM13 surpasses results that have been reported by other groups who have isolated and characterized fungal chitosan (12, 14, 16, 17, 21, 22). The quality of fungal chitosan obtained was dependent on the extraction protocol, pH and temperature applied during the extraction process, medium, carbon and nitrogen sources, as well as the concentration of nutrients used[16].

One main factor that influences yield is the C and N content as well as the concentrations of these substances. This factor does not only influence the yield but has been shown to be directly linked to the DD and MW of the chitosan obtained. The results from this study as in previous show that unlike nitrogen, higher concentrations of glucose did not result in higher yield of chitosan (results not presented)[12,13,14]. We believe that the catabolite repression induced by

the high concentrations of glucose may affect the enzymes involved in the synthesis of chitosan. In addition to nutrient content of the medium (i.e. content of trace elements) were found to be responsible in the catalysis of enzymatic processes. This suggests that the differences in medium composition and the presence of certain elements may affect the activities of chitin deacetylase [3,5,16] and therefore affect the DD of the chitosan. Therefore we may conclude that a carbon and nitrogen rich medium such as YPG is a more amenable medium for the production of chitosan when the concentration of readily assimilated carbon sources are kept below levels that cause catabolite repression. While TVB is defined as a growth medium that is rich in micro and macronutrients that is able to increasing the activities of enzymes involved in chitin and chitosan synthesis[5], in this particular study the defined medium was not as efficient in producing chitosan. Our results were in agreement with the report by Pochavanich and Suntornsuk (2002) who in his paper commented that higher nitrogen content increased the synthesis of enzymes involved in chitosan production[17]. However the sources of nitrogen and chitosan production will largely depend on the strain of fungi used[5].

The lower molecular weight observed in fungi grown on defined medium (TVB) may be due to the presence of elements such as ferum and manganese ions or certain substrates that may cause the activity of chitin synthase to slow down (5) or maybe even to activate the chitinase enzymes. The slowdown in the chitin synthase activities affects the building of chitin polymer chain, accounting for the derivation of relatively smaller chitosan polymers. The activation of chitinase on the other hand promotes the cleaving of chitin polymer chain and therefore shortening the length of the chitin polymer chain and reducing the molecular weight of the chitin and chitosan produced. LMW chitosan has been reported as having potential in antimicrobial and agricultural applications[6,9,13,15,20]. In addition the high levels of DD exhibited by the fungal chitosan was also indicative that the fungal chitosan that was produced in this research may be suitable for application in antimicrobial studies.

Both fungal and crustacean chitosan was used in the antimicrobial activities of chitosan. The antimicrobial activities were less prominent in the Gram negative bacteria (Table 7A and 7B) compared to the Gram positive. The polycationic nature of chitosan may be responsible for interaction with the electronegative bacterial cell surface. Thus, as suggested by Helander *et al.* (2001), the disruption of barrier properties of outer membrane of the bacteria may be the possible mechanism of antimicrobial action of chitosan[14].

The results also show that the higher concentrations of chitosan were not efficient in their inhibitory traits (Table 6 and 7) [8]. This we believe is due to the ability of chitosan to chelate nutrients, ions and trace elements. Chelation happens readily at liquid matrix (MHB) compared to solid (MHA). The solubility of chitosan will also affect the above and the ability to inhibit. The solubility of chitosan is higher at lower concentrations, and this we believe explains the efficiency of lower chitosan concentrations at inhibition[6,13].

In addition to examining the mechanism of inhibition on the microorganisms, the ultrastructural appearance of the microorganisms was examined via electron microscopy. In our examination of *Escherichia coli* and *Candida* spp grown on plates treated with chitosan, we found that the periplasmic space were abnormally expanded in our *E.coli* samples, while cellular degradation was observed in *Candida* spp isolated from plates treated with chitosan (results not shown).

The results obtained from this study indicates that the chitosan derived from the four isolates exhibited antimicrobial properties and produce higher levels of inhibition on Gram positive bacteria as compared to the negative. Further studies will be conducted on the antimicrobial properties of chitosan using chitosan salt derivatives from fungi to determine if the salts had higher inhibitory effects compared to the native form.

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